Reviving rare chicken breeds using genetically engineered sterility in surrogate host birds

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In macrolecithal species, cryopreservation of the oocyte and zygote is not possible due to the large size and quantity of lipid deposited within the egg. For birds, this signifies that cryopreserving and regenerating a species from frozen cellular material are currently technically unfeasible. Diploid primordial germ cells (PGCs) are a potential means to freeze down the entire genome and reconstitute an avian species from frozen material. Here, we examine the use of genetically engineered (GE) sterile female layer chicken as surrogate hosts for the transplantation of cryopreserved avian PGCs from rare heritage breeds of chicken. We first amplified PGC numbers in culture before cryopreservation and subsequent transplantation into host GE embryos. We found that all hatched offspring from the chimera GE hens were derived from the donor rare heritage breed broiler PGCs, and using cryopreserved semen, we were able to produce pure offspring. Measurement of the mutation rate of PGCs in culture revealed that 2.7×10^{-10} de novo single-nucleotide variants (SNVs) were generated per cell division, which is comparable with other stem cell lineages. We also found that endogenous avian leukosis virus (ALV) retroviral insertions were not mobilized during in vitro propagation. Taken together, these results show that mutation rates are no higher than normal stem cells, essential if we are to conserve avian breeds. Thus, GE sterile avian surrogate hosts provide a viable platform to conserve and regenerate avian species using cryopreserved PGCs.

conservation | biodiversity | primordial germ cell | poultry | genome editing

Cryopreservation of the oocyte and zygote is not possible in macrolecithal species, such as birds and fish, due to the large amount of lipid deposited in the female oocyte (1–3). For avian species, this signifies that, to conserve a breed of interest, birds must be maintained as extant breeding populations, which places them in danger to losses in biodiversity caused by population fluctuations and to the constant threat of extinction. The embryonic diploid reproductive germ cells from avian species offer an alternative means to cryopreserve the entire genotype of the germplasm (reproductive cells) from which a pure breeding population could be entirely reconstituted at a later date. This is a current research objective being pursued for both avian and fish species as a way to safeguard the genetic diversity of both farmed and rare/endangered breeds and species (2, 4, 5). The embryonic or primordial germ cells (PGCs) can be cryopreserved directly, or since their number is low in the early embryo (50 to 150 cells) (6–8), PGCs from a few select species can be propagated in culture to increase their number before cryopreservation (9–11).

The germ cell lineage is also believed to safeguard genetic information by having both high levels of homologous recombination and enzymes for DNA repair and by initiating programmed cell death when double-strand breaks are formed (12–16). The mutation rate of vertebrate germ cells in culture has not been measured but is thought to be low in comparison with somatic cell lineages, as intergenerational mutation rates are low in vertebrate species and during in vitro culture (17). Mammalian embryonic stem cells also have lower mutation rates in vitro (~100-fold) when compared with somatic cells (18).

Chicken is one of the few species from which PGCs can easily be propagated in vitro to increase cell number using a defined medium (19). For chicken, PGCs from a single embryo can be expanded in vitro to >100,000 cells within 4 wk and subsequently cryopreserved. Chicken PGCs can also be genetically modified during in vitro culture (9, 20–24). After thawing, PGCs transfer into the embryonic vascular system of "surrogate host" embryos, where they migrate to the forming gonads and will differentiate into functional gametes in the adult host (10, 25, 26). The adult surrogate host chickens are subsequently bred to generate offspring, some of which derive from the exogenous donor PGCs. Layer breeds of chicken, which have been selected for egg production, can be used as surrogate hosts for transplanted germ cells from other breeds of less fertile chicken and conceivably, from other avian species (27–29). A major constraint to the use of this system is that the transmission rate from exogenous PGCs injected into layer chicken embryos can vary greatly between individual surrogate host animals and between the different chicken breeds used as surrogate hosts (25, 30). To circumvent this problem, chemical and physical methods have been used to ablate the endogenous germ cells of the surrogate host and have

Significance

In the fields of conservation biology and sustainable agriculture, the ability to cryopreserve and revive animal species is paramount to efforts to preserve genetic diversity. An innovative approach is to use sterile surrogate host animals for the transplantation of reproductive germ cells from rare/endangered animals. This technology has previously been utilized in mammals but is of particular importance for animals with lipid-filled zygotes/embryos, such as fish and birds, which render cryopreservation techniques inefficient. We demonstrate that the female chicken rendered sterile using genome editing technology can be used as a surrogate host for transplanted cryopreserved germ cells and only lay eggs of the transplanted rare chicken breed. Our results suggest a way to preserve the biodiversity of bird species.

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been shown to increase the transmission of donor cell genetics. However, these agents are highly toxic to both the developing surrogate host embryo and the mature host animal (10, 31–33). Alternatively, the genetic ablation of a gene required for germ cell development has been used to eliminate the endogenous germ cells in both mammalian and fish species (34–38). These genetically sterile surrogate hosts were subsequently shown to efficiently generate offspring deriving from transplanted exogenous germ cells.

We recently used genome editors to disrupt the chicken *DDX4* (*Vasa*) gene, which is located on the Z sex chromosome in bird species (39). In birds, males are the homogametic sex containing ZZ sex chromosomes, whereas females are the heterogametic sex containing ZW sex chromosomes. In *DDX4* Z^- W mutant females, we observed that PGCs were reduced in number in the developing embryo and entirely absent in the posthatch ovary, leading to ovarian atrophy and a failure to lay eggs. Accordingly, the transfer of exogenous donor germ cells into *DDX4* Z^- W host females during embryonic development may rescue oocyte formation and restore egg production. Subsequent insemination of the DDX4 surrogate host with cryopreserved semen from the same donor breed would permit the complete reconstitution of the breed from frozen cellular material.

In this study, we demonstrate the reconstitution of a chicken breed from frozen cellular material. PGCs were first isolated from several rare traditional breeds of chicken and propagated in vitro to increase germ cell numbers before cryopreservation. We found that the genome of chicken PGCs was remarkably stable in culture: 0.65 de novo single-nucleotide variants (SNVs) were generated per cell division, giving mutation rates of 2.7×10^{-10}. Furthermore, endogenous avian leukosis virus, subgroup E (ALVE) retroviral insertions that are present in the chicken genome were not mobilized during in vitro propagation. *DDX4* Z^- W hosts formed oocytes from exogenous donor female germ cells isolated from a different chicken breed and all offspring derived from the donor PGCs. Donor PGC development was also sex restricted in *DDX4* Z^- W hosts; male PGCs did not generate viable oocytes indicating sex-restricted gamete differentiation in birds. Insemination of the *DDX4* Z^- W layer host with cryopreserved semen allowed for the complete reconstitution of a heritage broiler chicken breed. These results demonstrate the power of using sterile avian surrogate hosts for regenerating avian species.

Results

Propagation of PGCs from Rare and Heritage Chicken Breeds In Vitro. PGCs are present in the laid avian egg and reach a population of ~150 cells in the circulatory system of the chicken embryo at 60 h of incubation (6, 40). To expand this small population of cells, we sampled the embryonic blood from single embryos of a heritage broiler breed chicken. The Vantress heritage broiler chicken breed was first developed in the 1950s and maintained as a closed breeding population for the last 30 y (41). Embryonic blood was cultured in a defined medium containing either chicken serum or ovotransferrin (OT), an iron transporter supplement that can replace chicken serum in PGC culture media (19). PGC cultures were scored as positive if populations reached 50,000 cells within 4 wk of in vitro culture. Under this criterion, PGCs were successfully cultured in vitro from 40 to 56% of the embryos sampled (*SI Appendix*, Table S1). Primary cultures of female PGCs were obtained more efficiently using OT in place of chicken serum (61 vs. 30%), with an average in vitro doubling time of 33.4 h measured for both sexes. To extend these results to other chicken breeds, we obtained fertile eggs from several rare British chicken breeds (Cream Legbar, Marsh Daisy, Scots Dumpy, and Scots Gray) and assayed PGC growth using serum free medium containing OT. We successfully cultured both male and female PGCs from the majority of rare breed embryos sampled, with the derivation rate reaching 90% for some breeds (*SI Appendix*, Table S1). PGC cultures for all of these breeds were subsequently cryopreserved in vials containing 50,000 cells. PGCs survived cryopreservation well and proliferated robustly when recultured after thawing (*SI Appendix*, Fig. S1).

Germline Transmission Using Female Sterile Surrogate Hosts. Mouse embryonic stem cells and chicken PGCs have been shown to lose germline competence after extended periods in culture (42). To measure both germline transmission and loss of germline competence during in vitro propagation, we continuously propagated the heritage broiler breed PGCs in vitro for a total time of 3 or 6 mo before cryopreservation. We genetically labeled individual cultures of male or female PGCs with a fluorescent marker using piggyBac transposons containing either GFP (green; 3 mo) or tdTomato (red; 6 mo) fluorescent reporter genes early on during this culture period. Using labeled PGCs (Fig. 1 *A* and *B*) enables us to lineage trace the germ cells during embryonic development and to easily identify offspring deriving from the PGCs after injection into surrogate host embryos. Aliquots of frozen, labeled

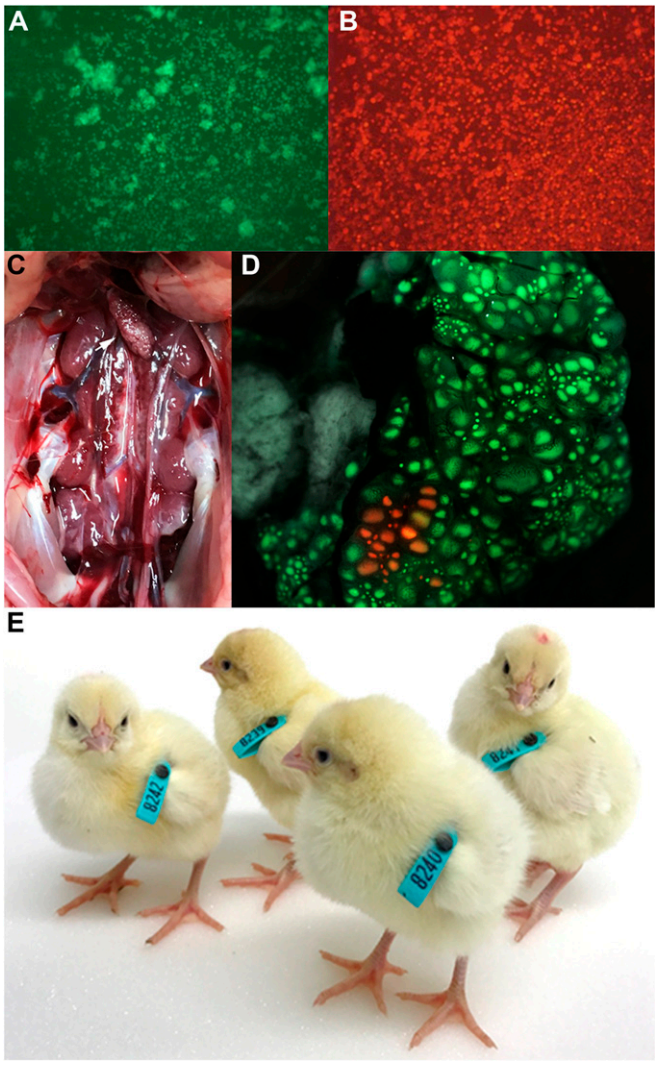

Fig. 1. Germline transmission using layer sterile surrogate hosts. (*A* and *B*) Vantress heritage broiler PGCs labeled with GFP or TdTomato fluorescent reporter transposons. (*C* and *D*) Ovary from a *DDX4* Z^- W hen at 8 wk posthatch injected with labeled PGCs. (*E*) GFP^+ offspring from *DDX4* Z^- W host hens. *DDX4* Z^- W hosts were artificially inseminated with layer semen, and hatchlings were screened for fluorescence.

donor PGCs (green, 3 mo and red, 6 mo) originally derived from a single male or female embryo were thawed and cultured for 1 wk and mixed in equal numbers, and ~4,000 total PGCs were injected into the dorsal aorta of day 2.5 host embryos. The host embryos were generated from crossing a *DDX4* ZZ^{-} male with wild-type (ZW) layer hens to produce host embryos composed of 4 genotypes: ZZ, ZZ^{-}, ZW, and Z^{-}W (39). Mixed male or female heritage PGC cultures were injected separately into host embryos. Injected embryos were hatched, and the founder host birds were raised to sexual maturity. Germline transmission from the donor PGCs was initially measured by mating the host birds and screening the offspring for GFP^{+} or tdTomato^{+} expression. As chromosomal integration events of transposon vectors are rare in cultured PGCs (1 to 3 insert events per transfection experiment), we expect to detect fluorescence in 50% of the offspring arising from a transposon-labeled PGC due to meiotic reduction (21).

Six experimental sets of embryo injections were carried out into a total of 91 fertile eggs obtained from a ZZ^{-} *DDX4* male to ZW wild-type female cross. From this number, 59 F0 founder hosts (65%) successfully hatched. Founder hosts genotyped as ZZ or ZZ^{-} injected with female PGCs were not bred, as female chicken PGCs have been previously shown to not form functional spermatozoa in male hosts (43, 44). It would be expected that females carrying a single Z chromosome will be sterile if the *DDX4* gene was knocked out and that ZZ^{-} males carrying a single-mutant *DDX4* allele would have normal fertility (39).

To assess germ cell colonization of the ovary of sterile females, the ovary from a *DDX4* Z^{-} W host injected with female donor PGCs was first examined at 8 wk posthatch (presexual maturity) (Fig. 1*C*). The ovary from this bird was entirely composed of fluorescent follicles, with the majority of the mature follicles being GFP^{+} and a small number of follicles being RFP^{+} (Fig. 1*D*). This result suggests that PGCs of a single genotype cultured in vitro for shorter time periods outcompeted PGCs cultured for longer periods in the developing ovary. The remaining 15 female birds were raised to sexual maturity to measure germline transmission of the donor female germ cells. Four of the 5 *DDX4* Z^{-} W host hens injected with female PGCs began to lay eggs when they reached sexual maturity at 22 wk posthatch and continued laying until 80 wk of age. Egg production was normal in these females, and no incidences of multiple ovulations (double-yolked eggs) were observed. Egg-laying measurements over a 2-mo period found that the injected *DDX4* Z^{-}W host hens were laying an average of 5.3 eggs per week, which was lower but similar to the injected *DDX4* ZW control host hens (6.6 to 6.8 eggs per week). The *DDX4* Z^{-} W hens were inseminated with wild-type layer semen at 24 wk of age, and the resulting offspring were analyzed for the fluorescent transgene by visual observation and PCR analysis (*SI Appendix*, Fig. S2); 280 eggs from 4 *DDX4* Z^{-} W hens (3 hens cohoused, 1 hen housed separately) were collected and incubated, from which 218 chicks hatched. Ninety-five of the offspring (44%) from the *DDX4* Z^{-} W hens were GFP or RFP fluorescent by visual observation and PCR positive for the transposon, indicating that they were derived from the donor heritage broiler germ cells (Fig. 1*E*, Table 1, and *SI Appendix*, Fig. S2) with an average transmission rate of 87%. Only 2 offspring were positive for RFP fluorescence. The fertility (percentage of day 18 eggs with embryos) was similar between the 4 *DDX4* Z^{-} *W* hosts and the 5 ZW wild-type hosts, signifying that ovulation and egg development proceeded normally in the *DDX4* Z^{-} *W* hens. Surprisingly, no fluorescent offspring were produced from ZW host hens, indicating that the donor female heritage broiler PGCs could not compete with the endogenous host germ cells in wild-type hens.

In *DDX4* Z^{-} *W* hosts injected with male donor heritage PGCs, none of the 5 *DDX4* Z^{-}W hens laid eggs. An analysis of the ovaries from these hens did not detect white or maturing yellow follicles (*SI Appendix*, Fig. S3). These results demonstrate that female heritage broiler donor cells could successfully generate offspring but only in the absence of endogenous germ cell competition, and male heritage broiler donor PGCs could not produce functional oocytes in female layer hosts, even with the absence of competing germ cells.

To measure male donor germ cell transmission, male ZZ or ZZ^{-} *DDX4* cockerel hosts injected with male heritage broiler PGCs were raised to sexual maturity and mated to wild-type females. Two of the 3 ZZ^{-} birds injected with male PGCs were crossed with wild-type layer hens after copy number PCRs showed high levels of GFP transgene DNA in their semen (*SI Appendix*, Fig. S4). However, no fluorescent offspring were observed, indicating that the heritage broiler PGCs were unable to compete with the endogenous layer male germ cells in a wild-type host (Table 1 and *SI Appendix*, Fig. S2).

To further verify the transmission rate from the *DDX4* Z^{-} W hens injected with donor female heritage broiler germ cells, we analyzed the offspring at embryonic stages. Embryo analysis revealed that slightly higher numbers of the embryos were GFP^{+} (46%; 92% transmission rate), which suggests that the lower germline transmission rate observed in hatchlings could be due to the toxicity of the transposon insertion or that some offspring were derived from endogenous host oocytes (*SI Appendix*, Fig. S5 and Table S2). To accurately determine the pedigree of the offspring arising from the *DDX4* Z^{-} W host hens, a principal component analysis (PCA) of genetic variation was performed on genomic DNA from offspring, surrogate host brown layer chicken, and control heritage broiler chicken using a 60,000 chicken single-nucleotide polymorphism (SNP) genotyping assay

Table 1. Germline transmission rates from host hens injected with donor Vantress heritage broiler PGCs

Host genotype	Sex of injected donor heritage PGCs	No. of host birds	No. of fertile host birds	No. of eggs laid per week per host hen*	No. of eggs incubated	Fertility† (% of eggs incubated)	No. of chicks hatched (% of eggs incubated)	No. of chicks GFP^{+}	No. of chicks RFP^{+}	% Transmission‡
ZW	♀	3	3	6.6	206	175 (85)	146 (71)	0	0	0
ZW	♂	2	2	6.8	175	146 (83)	144 (82)	0	0	0
Z^{-}W	♀	5	4	5.3	280	242 (86)	218 (78)	93	2	87
Z^{-}W	♂	5	0		Not laying					
Z^{-}Z	♂	2	2		378	363 (96)	321 (85)	0	0	0

The number of hosts injected with male or female PGCs for each donor genotype is shown, with numbers of eggs incubated and offspring hatched for each genotype.

*Laid eggs were counted during a 60-d period when hens were between 7 and 10 mo of age and divided by the number of fertile hens present in pen. The maximum possible lay rate is 7.0 eggs per week.

†Fertility: number of embryos detected by candling eggs at day 18 of incubation.

‡% Transmission: the number of GFP^{+}/RFP^{+} chicks per number of hatched chicks equals one-half the transmission rate due to meiotic reduction.

(Fig. 2). We found that all offspring from *DDX4* Z^- W surrogate hosts injected with heritage broiler donor PGCs clustered between the heritage broiler and brown layer host bird groupings, indicating their hybrid origin. Offspring from ZW host birds clustered with the ZW surrogate hosts, indicating that they were offspring of the host endogenous germ cells. These results indicate that all offspring from the *DDX4* Z^- W host hens were derived from donor heritage PGCs.

As we determined that all offspring of the *DDX4* Z^- W host hens were derived from the cryopreserved donor germ cells, it would be possible to use cryopreserved heritage broiler semen, although an inefficient procedure for many chicken breeds, to regenerate pure heritage broiler offspring. To demonstrate that a pure chicken breed can be reconstituted from cryopreserved reproductive material (i.e., germplasm), we first artificially inseminated the *DDX4* Z^- W host females with fresh semen from heritage broiler cockerels, and 3 putative pure heritage broiler birds were produced (Fig. 3*A*). We next cryopreserved semen from a single heritage broiler cockerel and used this to inseminate a single *DDX4* Z^- W female. Fertile eggs from this mating were obtained and incubated to hatch, and a single putative pure heritage broiler bird was produced (Fig. 3*B*). A PCA of these 4 offspring demonstrated that they clustered with Vantress heritage chicken, confirming that they were reconstituted pure heritage broiler offspring (Fig. 2). Examination of GFP fluorescence and PCR analysis confirmed the presence of the GFP transgene in several of these offspring (*SI Appendix*, Fig. S6). Thus, it is possible to regenerate a breed of chickens from cryopreserved cells in a single step using sterile surrogate host hens (Fig. 4).

Genome Stability of In Vitro Propagated PGCs. To assess genomic stability, a karyotype analysis of the chromosomal complement is usually informative. In avian species, this analysis is difficult due to the characteristics of the avian karyotype: the presence of a few macrochromosomes and many microchromosomes. For example, the chicken has a diploid number of 78 chromosomes: 10 pairs of macrochromosomes, 28 pairs of microchromosomes, and a pair of highly dimorphic sex chromosomes (45). A alternative approach is to examine the formation of de novo SNV and transposable element mobilization during in vitro propagation. We analyzed SNV formation in PGCs propagated from single cells, as de novo SNVs might be undetectable when formed in larger cell populations. PGCs derived from individual embryos were cultured for 55 d, and then, single PGCs were isolated and expanded clonally to generate sufficient genomic DNA for whole-genome resequencing (WGS). We compared the WGS data from 8 single-cell clones (derived from 4 male and 4 female PGC cultures) with WGS data from the original embryo and found that the average de novo SNV formation was 25.8 SNVs ± 9.4 SD formed during 55 d in culture, which was equivalent to 39.6 cell generations (Fig. 5). PGC cultures, therefore, have a low mutation rate of 0.65 SNVs per cell per generation (2.7×10^{-10} per nucleotide per generation). Nine of a total of 140 SNVs (6.4%) were located in exonic sequences. On average, after 55 d in culture, only 1 coding mutation was found in the genome of each clone, none of which generated premature truncations (Dataset S1). We used the same WGS data from the 8 single-cell clones to analyze chromosomal coverage by read depth over the entire mapped genome and compared this coverage with the WGS data from the original embryo. Differences in coverage between chromosomes for the single-cell clones when compared with coverage between chromosomes for the respective embryonic genomic sequence would indicate major chromosomal duplications/losses. This analysis indicated that the normal chromosomal complement was present in the cultured PGCs with no major aneuploidy events (*SI Appendix*, Fig. S7).

ALVE retrotransposons are evolutionarily recent retrotransposable elements found only in the genomes of the domestic chicken and its wild progenitor, the red jungle fowl (46). As such, these elements typically retain the ability to retrotranspose, moving and propagating by "copy and paste" across the genome, particularly during periods of cellular stress, and this can lead to disease

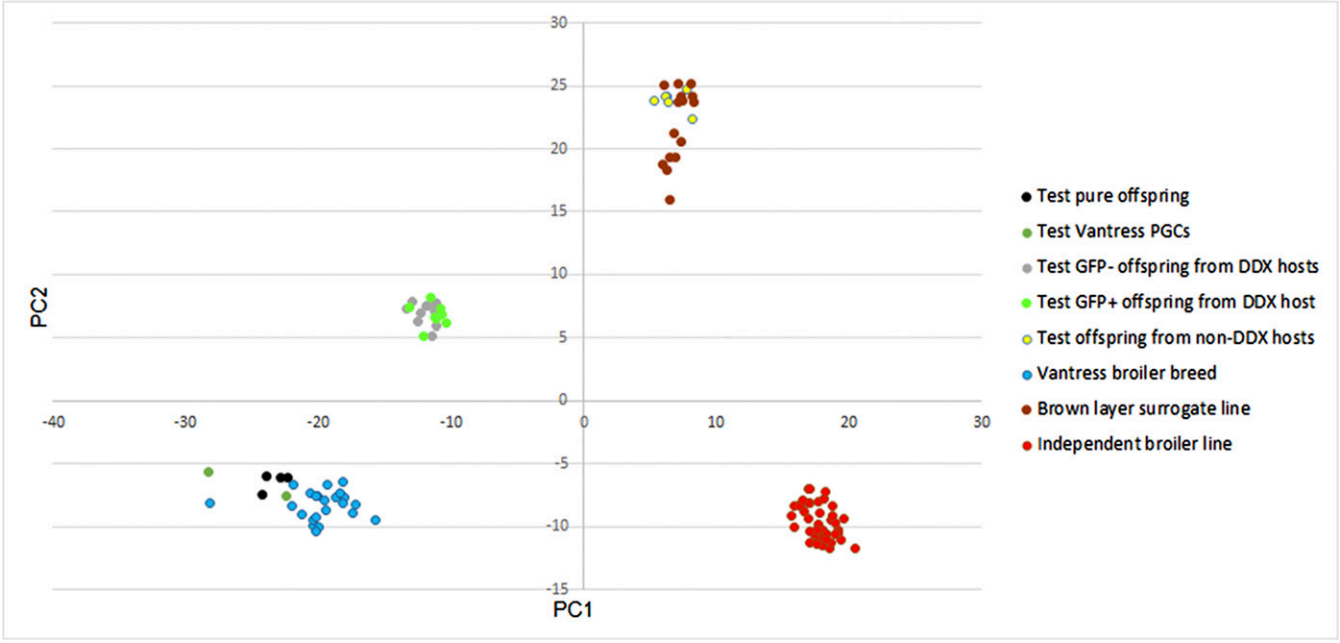

Fig. 2. Principal component (PC) analysis of offspring from DDX4 Z^- W and ZW surrogate hosts. DNA samples were genotyped on a 66,000-SNP chip and analyzed for PCs. Three chicken breeds were analyzed: the Vantress heritage breed (blue), an independent pedigree broiler line (red), and the Hy-Line brown layer *DDX4* surrogate host line (brown). Offspring (green and gray) from the *DDX4* Z^- *W* hosts clustered between the Vantress breed and the Brown layer line. Offspring (black) from a *DDX4* Z^-W host inseminated with Vantress semen clustered with the Vantress breed chicken.

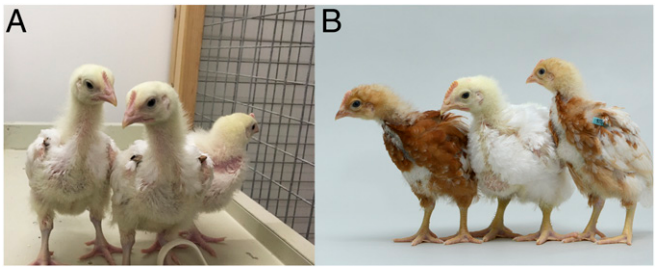

Fig. 3. Pure offspring produced from DDX4 Z^- W hens using heritage broiler semen. (*A*) Fresh or (*B*) cryopreserved semen pooled from 3 adult males was used to fertilize a *DDX4* Z^- W founder female. The pure line Vantress chick in *B* is shown surrounded by 2 control layer offspring.

outbreaks (47–49). To measure the stability of endogenous ALVEs, we first mapped these endogenous ALVEs in the Vantress heritage broiler breed. We then compared the location of the ALVE inserts between individual embryos and PGCs isolated and cultured from those embryos both in total PGC populations and after single-cell isolation and propagation. A total of 13 different ALVEs were identified across the 24 WGS datasets (*SI Appendix*, Table S3). Twelve of these elements were previously identified within broilers (including a heritage Cobb dataset), with the 13th (ALVE_ros304; 1:122,259,275; CACAGG) characterized in this study. Individual ALVE frequencies ranged from 0.05 to 0.85, and only ALVE_ros304 was present in all samples. All ALVE identification and genotype results matched exactly between the embryo, PGC cultures, and clonally propagated PGC data, suggesting that there was no allelic dropout or additional integration events after initial isolation and culture of the embryonic PGCs. It is, however, possible that additional integrations could have occurred in poorly assembled regions of the genome and thus, could be undetectable by our analysis. In summary, we carried out 3 independent measures of genome stability and identified no major instabilities during the in vitro propagation of PGCs.

Discussion

Diploid PGCs have the potential to be used for cryopreservation of bird species. Successful biobanking of birds using PGCs will have important applications for both ex situ conservation of endangered bird populations and conservation of biodiversity in both commercial and indigenous chicken breeds. We and others have demonstrated that PGCs from various commercial chicken breeds can be cultured for periods of several weeks before cryopreservation, and when thawed, they will migrate to the forming gonads of a surrogate host embryo and contribute to the genome of offspring (25, 50). Our study demonstrates that PGCs can be cultured from most rare chicken breeds, and the donor germ cells from a heritage broiler breed can rescue follicle development in the germ cell ablated layer hens, leading to normal egg production and viable offspring (Fig. 4). We could not demonstrate germline transmission from the PGCs of these other rare chicken breeds, as they were raised under "free range" conditions and are of unknown immunological status. However, our success with the heritage breed suggests that this methodology could be extended to all chicken breeds.

In general, donor chicken PGCs can contribute to the germline of wild-type surrogate hosts with varying efficiencies (10, 25, 26). We would have expected to see some level of germline transmission from the wild-type surrogate hosts used in these experiments. This loss of transmission implies that germ cells lose their ability to compete with endogenous germ cells when they are propagated in culture for at least 3 mo or that PGCs cultured from the broiler heritage line are unable to compete with endogenous germ cells in layer hens and layer roosters. However, only 2% of the offspring generated from Vantress heritage PGCs were derived from cells cultured for 6 mo in comparison with 98% of the offspring from cells of the same genotype cultured for 3 mo. This suggests that the competence to compete for the gonadal niche is lost with cellular divisions. This loss of competence was first observed in the developing ovary of an 8-wk posthatch *DDX4* Z^- W host. Further study of gonad development in the surrogate hosts is needed to determine when the contribution of germ cells to the follicular pool is determined. It is also possible that the defined medium using Activin A and FGF2 used to culture the PGCs does not reflect the in vivo gonadal niche and needs to be further optimized (19). Surprisingly, we did not observe germline transmission from male PGCs transplanted into female sterile hosts as has been previously reported by us and others when host sterility was induced using chemical means or irradiation (51, 52). We suggest that this observed difference may be attributed to either the breed of the donor male PGCs used in these experiments and/or the fact that the *DDX4* Z^- W female contains endogenous PGCs at prehatch stages, which may compete with the donor male germ cells. Differences in germline transmission of donor PGCs injected into host embryos of different breeds have been previously observed (30). Our data suggest that the heritage broiler breed PGCs did not compete efficiently with endogenous PGCs in the wild-type layer host gonad. The future use of sterile surrogate host embryos should circumvent this issue of donor/host competition. Our results highlight the requirement to couple semen cryopreservation programs with PGC cryopreservation. Alternatively, the development of a sterile male surrogate host would permit rare breed regeneration using only cryopreserved PGCs.

In this study, we sought to determine the effect that propagating PGCs in culture has on their ability to contribute to the germline, as loss of germline competence is observed with increasing periods of in vitro culture. Our experiments clearly demonstrated this effect. By challenging cells of a single genotype in a single host, we demonstrated that PGCs cultured for less time better competed for the stem cell niche and gave rise to more offspring—although these experiments did only use a single PGC line. It is notable that the replacement of chick serum with OT in the culture media significantly improved the rate of female PGC culture derivation for the Vantress heritage line. Chick serum contains numerous amino acids, lipids, cytokines, and growth factors among other components. Currently, it is unclear which of these factors may be detrimental to establishing female PGC cultures or whether any serum components may adversely affect germline transmission or mutation frequency. Furthermore, cryopreservation of PGCs may also alter their competency in this context, and it would be interesting to investigate the effect that freezing and thawing cultures has on transmission. Additionally, we measured the de novo mutation

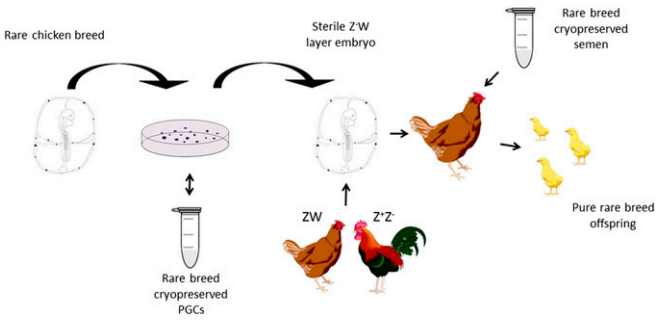

Fig. 4. Diagram of the reconstitution of a poultry breed using cryopreserved cells. Cryopreserved female rare breed PGCs introduced into a sterile surrogate host hen inseminated with frozen rare breed semen will produce "pure" rare breed offspring.

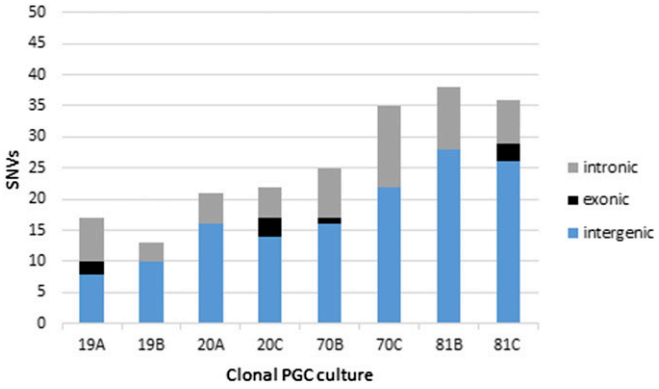

Fig. 5. Total SNVs mapped in 8 clonal cell lines compared with somatic embryonic DNA. PGCs were cultured from individual embryos and then cultured clonally after being propagated 55 d in culture. PGC DNA was compared with somatic DNA from the original embryo. Lines 19 and 20, male PGCs; lines 70 and 81, female PGCs. A, B, and C suffixes indicate individual clonal populations derived from each PGC line.

rate of PGCs during in vitro propagation. The number of SNV mutations that we detected in these experiments is approximately 1 every 2 doubling times or 1 every 3 d in culture. Somatic cells in culture have been shown to have a mutation rate of 4.7×10^{-8} per division for normal human fibroblasts (53). The mutation rate of mouse embryonic stem cells is thought to be low ($<1 \times 10^{-9}$) (18). The mutation rate measured here was similar to that shown for embryonic stem cells but not as low as for human and mouse intergenerational mutation rates (17, 18). Lynch (54) estimated the intergenerational mutation rate of 7.7×10^{-10} per site for somatic cells and 6.0×10^{-11} per site in the human germline. We also determined that the endogenous ALVE elements were stable in PGCs during extended in vitro culture. Both results suggest that in vitro expansion of avian PGCs for periods up to 3 mo to increase cell number is not detrimental to the birds generated from these cells. In fact, sufficient PGCs for cryopreservation and regeneration of a breed can be generated within 5 wk of in vitro cell culture.

Our results demonstrate the power of using sterile surrogate host hens for reconstituting chicken breeds from frozen material (Fig. 4). Our methodology clearly demonstrates the benefits of using genome editing technology to generate surrogate host chickens for the preservation of valuable chicken breeds and aid efforts to conserve genetic diversity. This work reflects recent efforts to use genetic modification to ablate the endogenous germline in other species, such as fish and mammals (36, 55–57). This study also points to the need to determine if it will be possible to generate offspring of multiple individual genotypes from single surrogate chickens that have been injected with PGCs cultured from several embryos. As cryopreservation of poultry semen is problematic and varies in success between chicken breeds (58, 59), the generation of male sterile chicken will bypass the need for semen cryopreservation and permit the resurrection of a poultry breed in a single cross of surrogate host animals.

Methods

Animal Husbandry. The *DDX4* line of knockout chicken was maintained on a Hy-Line Brown layer background. *DDX4* ZZ^- cockerels were mated with wild-type Hy-line hens to generate fertile eggs for injection and hatching and additional ZZ^- cockerels for line maintenance. Marsh Daisy, Cream Legbar, Scots Dumpy, and Scots Gray eggs were sourced from local UK poultry breeders. Fertile Vantress heritage broiler eggs were obtained from the Vantress heritage flock kept by Cobb-Europe at the Colchester UK facility. Germline transmission experiments and the *DDX* flock maintenance were conducted under UK Home Office license and regulations. The experimental protocol and studies were reviewed by the Roslin Institute Animal Welfare and Ethical Review Board Committee.

Culture and Transfection of PGCs. PGC derivation and propagation were carried out as described in ref. 19. Briefly, 1 μL of blood isolated from a stage 15 to 16^+ HH embryo was placed in culture medium containing 1× B-27 supplement, 0.15 mM $CaCl_2$, 2.0 mM GlutaMax, 1× nonessential amino acids (NEAA), 0.1 mM β-mercaptoethanol, 1× nucleosides, 1.2 mM pyruvate, 0.2% ovalbumin (Sigma), 0.01% sodium heparin (Sigma), 4 ng/mL FGF2 (R&D Biosystems), and 25 ng/mL Activin A (Peprotech) in Avian Knockout Dulbecco's Modified Eagle Medium (DMEM) (250 osmol/L, 12.0 mM glucose, containing no calcium chloride; Thermo Fisher Scientific; custom modification of knockout DMEM). Either 5 μg/mL OT (Sigma) or 0.2% chicken serum was added to the final culture medium. Female and male PGC cultures were derived from the Vantress heritage broiler line or rare breed chicken embryos; frozen in avian knockout DMEM containing 4% dimethyl sulfoxide (DMSO), 5% chicken serum, and 0.15 mM $CaCl_2$ an average of 4 wk after derivation in aliquots of 50,000 PGCs; and stored at −150 °C. PGC cultures were frozen at least once before injections into surrogate host embryos or used for clonal DNaseq analysis; 50,000 cells were resuspended 250 μL serum/DMSO freezing mix in polypropylene cryovials. Cells were frozen in a −80 °C freezer in an isopropanol jacket (Mr Frosty). Cryovials were stored at −150 °C after overnight freezing. PGCs and embryos were sexed using W chromosome-specific primers as described in ref. 10.

To fluorescently label cells for germline transmission, PGCs (~2.0×10^5 cells and 6 wk in culture) from the 81 (female) or 19 (male) PGC cultures were transfected using 3 μL of DIMRIE-C (Life Technologies) with 2 μg of the piggyBac Hybase transposase and 2 μg of the transposon vector piggyBac-CAG-GFP-IRES–puromycin (Macdonald, 2012) or piggyBac-CAG-TdTomato-IRES–puromycin and selected with 0.5 μg/mL puromycin starting at 4 d posttransfection for 2 wk. All PGCs were visibly fluorescent. Labeled PGCs were continuously propagated at 1.0 to 4.0×10^5 cells per 1 mL, with media replaced every 2 d until total time in culture reached either 3 or 6 mo, at which point cultures were frozen and stored at −150 °C.

Germline Transmission. GFP^+ (cultured for 3 mo) and $TdTomato^+$ (cultured for 6 mo) from either the E81 (female) or the E19 (male) PGC culture were thawed and cultured for 4 to 8 d. Then, they were mixed 1:1; 1 μL of medium containing 5,000 to 7,000 female or male PGCs was injected into the dorsal aorta of stage 16^+ HH embryos generated from crosses between Z^-Z males with a single *DDX4* allele (39) and wild-type Hy-Line Brown layer hens (ZW). Eggs (ZZ, ZZ^-, ZW, Z^-W) were windowed at the pointed end before injection, and 50 μL of 1× penicillin/streptomycin was injected into the cavity before resealing with shell membrane and melted parafilm. Seven injection experiments were carried out, and founders were screened by PCR for the presence of the GFP transgene to determine if they were Z^-Z, Z^- W, or wild-type (ZZ or ZW) for the *DDX4* allele. To calculate germline transmission of injected PGCs, female founders were artificially inseminated with wild-type Hy-Line semen, and founders were screened for fluorescence using Headsets (Biological Laboratory Instruments). Both GFP^+ and negative offspring were screened by PCR using primers for the GFP (ACGTAAACGGCCACAAGTTC, AAGTCGTGCTGCTTCATGTG) and GAPDH (CAGATCAGTTTCTATCAGC, TGTGACTTCAATGGTGACA) to confirm transmission results. Semen from male founders was screen by PCR for the presence of the GFP gene as described in ref. 60 using transposon-specific primers (CACACCGGCCTTATTCCA, CAACGAGAAGCGCGATCACAT). Males were then individually housed with 4 Hy-Line Brown layer hens for natural mating. Additional eggs from founders that were not taken to hatch were windowed between days 3 and 5 of development, and fluorescence was observed using a Zeiss AxioZoom.v16 microscope. Statistical analysis of germline transmission was carried out using Fisher's exact test, with significance taken as $P < 0.05$.

Semen Freezing. Semen was collected from broilers by the method of abdominal massage (61). Semen was diluted with 2.5 vol of extender (2.85 g sodium glutamate, 0.5 g glucose, 0.25 g inositol, 0.5 g potassium acetate, 0.07 g magnesium acetate-$4H_2O$ in 100 mL sterile water, pH 7.0), cooled to 4 °C, and supplemented with 6.5% dimethylformamide as cryoprotectant. The semen/cryoprotectant mixture was loaded into 0.5-mL straws (IMV), heat sealed, and frozen on a rack 4 cm above the surface of liquid nitrogen in an insulated container. The frozen straws were stored in liquid nitrogen. Straws were thawed in a water bath at 4 °C and then, inseminated into the everted oviduct of the recipient hen. Eggs were collected daily, and batches of eggs were set for hatching once per week.

Immunohistochemistry. Tissues were fixed in formalin for paraffin sections followed by hemotoxylin/eosin staining or cryoembedded and processed for immunofluorescence (19).

Genomic DNA Extraction. Tissue from the stage 15 to 16^{+} HH embryos sampled to derive the Vantress heritage PGC cultures was removed from eggs and stored at −80 °C. Tissue was thawed, placed in lysis buffer (400 mM Tris·HCl, pH 8, 60 mM ethylenediaminetetracetic acid (EDTA), 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 100 μg/mL Proteinase K; Thermo Fisher Scientific), and incubated with gentle agitation at 55 °C for 3 h. Lysed tissues were centrifuged (1 min at 13,000 × *g*), and the supernatant was added to an equal volume of phenol/chloroform/isoamyl alcohol, mixing gently by inversion for 30 s. The emulsion formed was centrifuged (10 min at 12,000 × *g*), and the aqueous phase was added to an equal volume of chloroform and mixed by inversion; then, centrifugation was repeated. The aqueous phase was added to 0.8 vol of isopropanol to precipitate genomic DNA; then, centrifugation was repeated, and 0.8 mL cold 70% EtOH was added to wash. Supernatant was removed after a final centrifugation, and the DNA was air dried for 5 to 10 min and dissolved in 30 μL of 10 mM Tris, 1 mM EDTA (TE) buffer.

For cultured PGCs, genomic DNA was extracted from 1.0 to 2.0 × 10^6 cells using the Gentra Puregene Cell Kit (Qiagen; catalog no. 158722) according to the manufacturer's instructions. Air-dried DNA was dissolved overnight at 4 °C in 50 μL of 1× TE buffer. Quality of genomic DNA was confirmed by Nanodrop and running 1 μL of genomic DNA on a 0.8% agarose gel.

Pedigree Analysis Using SNP Chips. Genomic DNA was prepared from blood or chorioallantioic membrane samples from G_1 chicks using cell lysis solution (Qiagen) and RNase A Solution (Sigma). Protein Precipitation Solution (Qiagen) was added, and DNA was precipitated and resuspended. DNA from these G_1 chicks and DNA from control chickens (pure line commercial broiler, Vantress breed, putative hybrids, and control brown layer Hy-line flock) were genotyped using a custom Cobb 60K Infinium Illumina array. A PCA was then completed using 60,000 genotypes from each of the base populations.

Sequencing and Variant Analysis. Cryopreserved cells for PGC cultures 19, 20, 70, and 81 were recultured for a total of 55 d, at which point single PGCs were transferred into 96-well plates. These single cell clonal cultures ($n = 2$) were propagated until cell number reached 4.0 × 10^5 cells per 1 mL at which point 1.0 × 10^5 cells were isolated approximately every 2 d and pelleted by centrifugation ready for genomic DNA extraction and WGS.

Whole-Genome Resequencing. Short read WGS was performed by Edinburgh Genomics. Embryo genomic DNA sample libraries were prepared using the Illumina TruSeq DNA PCR-free, gel-free protocol with average insert sizes of 550 bp and sequenced using the Illumina HiSeq 2500 instrument generating 250-bp paired end (PE) reads. PGC genomic DNA samples were prepared later, again using the TruSeq library preparation but with average insert sizes of 350 bp, and sequenced using the Illumina HiSeqX platform generating 150-bp PE reads.

De Novo Variant Calling. Raw sequencing data were quality checked using FastQC Screen (62), and Illumina PE TRuSeq3-2 adapter sequences were removed using Trimmomatic v0.36 (63). Data were aligned to the chicken reference genome (Gallus_gallus_5.0; GenBank accession no. GCF_000002315.4) using BWA-mem v0.7.15 (64). Alignment files were analyzed and improved using PicardTools v2.9.4 (65) and GATK v3.7 (66) following the GATK best practices pipeline, and variants between the embryo and PGC sequencing data were called using Mutect2.

To distinguish between germline variants and de novo variants occurring during cell culture, variants were retained as long as no alternative allele reads were present in the embryo sample from which the PGCs were isolated, allele read depth was at least 15, heterozygous alleles in the PGC sample had frequencies within a 95% confidence interval and were not listed in the dbSNP chicken database, and reads supporting variants were uniquely mapping. Only reads aligned to chromosomes 1 to 28, 30, 33, the mitochondrial genome, and the sex chromosomes were used for calling de novo variants. SNV rates were calculated as measured events per diploid avian genome containing 2.4 × 10^9 bp (Gallus_gallus_5.0; GenBank) and an average doubling time of 1.39 d (4 Vantress PGC lines measured in 3 biological replicates).

Chromosomal Analysis. The coverage information was extracted from bam files in bedgraph format using the genomecov function in Bedtools 2.26.0. The sequence data were checked to ensure that the clonal lines have representation (i.e., coverage) of all of the chromosomes, as that would indicate there was no loss of entire chromosome during the culture process of the PGCs. Moreover, mean depths of sequencing coverage for each chromosome were compared between the clonal lines and their respective embryos in order to check if there was any major shift in overall coverage, as that might indicate possible loss or gain in part of a chromosome.

Detection of ALVE Integration Sites. ALVE integrations were identified in the embryo, PGC, and clonal PGC WGS data using the obsERVer identification pipeline on Illumina PE whole-genome resequencing data. Data were from 10 heritage broiler embryos (E5, E13, E19, E20, E27, E48, E62, E70, E81, and E90), their matched PGC cultured isolates, and clonal PGC populations originating from PGC cultures 19, 20, 70, and 81. Briefly, obsERVer identifies ALVE integrations by aligning reads to an artificial pseudochromosome constructed of reference ALVE sequences and then, aligns clipped reads and their mates to the Gallus_gallus5.0 reference genome sequence (GenBank accession no. GCF_000002315.4) to identify integration junction sites. Putative sites were validated by the clipped ALVE integration sequence and by comparison with the previously identified sites pipeline.

Identified ALVEs were genotyped directly by mapping reads to the reference genome assembly with BWA-mem v0.7.10 (64) and manipulating the map files with samtools v0.1.19 (67). Results from all matched datasets were compared to provide a measure of genetic stability following PGC culture from original embryonic tissue. ALVE sequence from clipped integration junction reads was used to identify terminal truncations and ALVE orientation.

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